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Fig. 1.

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Parvovirus 4 in French in-patients:

A study of haemodialysis and lung transplant cohorts.

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Running head: Parvovirus 4 in French in-patients.

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ABSTRACT

The epidemiology and the clinical implication of human parvovirus 4 (PARV4) in human populations is still under evaluation. The distribution of PARV4 DNA was determined in cohorts of French haemodialysis and lung transplant patients. Plasma samples (n=289) were tested for PARV4 by real-time PCR assay (ORF2), and amplification products selected at random were sequenced. Analysis of available serological and biological markers was also undertaken. Fifty-seven samples out of 185 (30.8%) were positive for PARV4 DNA in the cohort of haemodialysis patients. A higher prevalence of the virus was identified in patients with markers of HBV infection. PARV4 was also identified in 14 out of 104 samples (13.5%) from lung transplant recipients, with no clear-cut association with available clinical markers. Point mutations located on the zone of real-time detection were identified for some amplification products. This study describes the detection of PARV4 in the blood of haemodialysis and lung transplanted patients with significant difference in prevalence in these two cohorts. Further studies will be needed in order to understand better both the potential implication in host health and the natural history of this virus.

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KEY WORDS: PARV4, *Parvoviridae*, haemodialysis, lung transplant, epidemiology,
genetic diversity.

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INTRODUCTION

Parvovirus 4 (PARV4) is a virus discovered recently belonging to the family *Parvoviridae* [Jones et al., 2005]. Identified initially in the blood of a patient harbouring acute viral syndrome, this virus had limited sequence homology with parvovirus B19 (<30% aa similarity) despite a conserved genomic organization showing two large non-overlapping ORFs [Simmonds et al., 2008]. First studies revealed that the virus was present in the blood from intravenous drug users and individuals positive for HCV or HIV at prevalence ranging from 6% to 30%, and in cohorts of kidney transplant patients as well [Biagini et al., 2008; Fryer et al., 2006; Fryer et al., 2007; Lurcharchaiwong et al., 2008; Vallerini et al., 2008]. PARV4 had been also identified in individuals without apparent pathology like blood donors (1%-24%), and in blood products negative for parvovirus B19 DNA [Fryer et al., 2007; Lurcharchaiwong et al., 2008; Touinssi et al., 2010; Vallerini et al., 2008]. Genetic diversity of the virus is poorly known, but was extended recently to highly divergent animal variants identified in domestic (cow, pig) and wild (baboon, chimpanzee) species [Adlhoch et al., 2010; Lau et al., 2008; Sharp et al., 2010]. Interestingly, these studies demonstrated high prevalence values (up to 40%) for these PARV4-related animal viruses.

Another member of *Parvoviridae* family identified recently, human Bocavirus (HBoV) [Allander et al., 2005], had been more investigated and is suspected to be associated with wheezing and respiratory disease, mainly in young children [Brown, 2010].

Despite these advances, the natural history and the clinical role of these new viruses, and more particularly that of PARV4, remain unknown to a large extent.

Aim of this study consisted in the evaluation of PARV4 DNA prevalence in two different cohorts of haemodialysis patients and lung transplant recipients, combined with the analysis of available serological and biological markers. An additional investigation of HBoV DNA in lung transplant patients was also performed.

MATERIALS AND METHODS

Clinical samples

Two hundred and eighty-nine plasma samples originating from two cohorts of in-patients from south-eastern France were analyzed retrospectively:

- i) 185 haemodialysis patients (mean age 64 years; sex ratio 1.37 (men:women), mean duration of dialysis 30 ± 28 months) from the Nephrology and Renal Transplant Centre, Conception Hospital (Marseille) (date of sampling: 2007);
- ii) 104 adult lung transplant recipients (mean age 42 years, sex ratio 0.73) transplanted between October 2005 and August 2008, and followed at the Lung Transplant Centre of the University Hospital of Marseille (date of sampling: from June 2007 to December 2008); blood samples were not available before transplantation.

Information relating to the presence of specific antibodies to HCV, HBV, HIV, and HBs antigen in the plasma samples tested was collected. Of the 185 haemodialysis patients, 22 tested positive for HCV, 75 for HBV, 4 for HIV, and 5 for HBsAg. Of the 104 lung transplant recipients, only 2 tested positive for a serological marker of HCV.

Blood samples were collected in vacuum tubes (Vacutainer, SST, Becton Dickinson, Meylan, France), centrifuged, and plasma aliquots were stored at -80°C

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3 98 prior to DNA extraction. Total nucleic acids were extracted from 1 ml of plasma by
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5 99 using the MagNA Pure LC instrument (Roche Diagnostics, Meylan, France), as
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8 100 described previously [Biagini et al., 2008; Touinssi et al., 2010].
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13 102 **PARV4 PCR assay**
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15 103 Samples were screened for the presence of PARV4 DNA by real-time PCR
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17 104 (StepOne Plus, Applied Biosystems, Courtaboeuf, France) using a TaqMan detection
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20 105 system located on the ORF2 of the viral genome. Primers used were PARV4Fwd (5'-
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22 106 CTAAGGAAACTGTTGGTGATATTGCT-3') and PARV4Rev (5'-
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24 107 GGCTCTCCTGCGGAATAAGC-3'), in combination with fluorogenic probe PARV4-N
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27 108 (5'-FAM-TCCTACYGCCCCTCCTCCTTCTT-TAMRA-3') [Touinssi et al., 2010].
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29 109 Amplification reactions were performed using 5 µl of extracted nucleic acids in
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32 110 a 20 µl reaction volume (TaqMan Fast Universal PCR kit, Applied Biosystems)
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34 111 according to manufacturer's instructions. The amplification conditions were 95°C for
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36 112 20 seconds, followed by 50 cycles of 95°C for 1 second and 60°C for 20 seconds.
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39 113 The sensitivity of the TaqMan assay was estimated at 10 copies of PARV4
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41 114 DNA per reaction using dilutions of a synthetic template corresponding to the target
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43 115 sequence (103 nt) [Biagini et al., 2008; Touinssi et al., 2010].
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48 117 **HBoV PCR assay**
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50 118 A real-time PCR assay for HBoV DNA located on the non-structural protein
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53 119 (NP-1) gene was further applied to samples belonging to the lung transplant cohort.
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55 120 Primers used were STBoNP-1f (5'-AGCATCGCTCCTACAAAAGAAAAG-3') and
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58 121 STBoNP-1r (5'-TCTTCATCACTTGGTCTGAGGTCT-3'), in combination with
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122 fluorogenic probe STBoNP-1pr (5'-FAM-AGGCTCGGGCTCATATCATCAGGAACA-
123 TAMRA-3') [Tozer et al., 2009].

124 Amplification conditions were identical as those adopted for PARV4 detection.
125 The detection limit of the real-time PCR assay was 10 copies of HBoV DNA per
126 reaction [Tozer et al., 2009].

128 **Sequence analysis**

129 Real-time PARV4 and HBoV amplification products (from negative or positive
130 samples, n=15 when available) were chosen at random in both cohorts tested, and
131 analysed by agarose gel electrophoresis. Positive PCR products were excised and
132 purified from agarose (Qiaquick Gel extraction kit, Qiagen, Courtaboeuf, France),
133 cloned into the pGEM-T Easy Vector System (Promega, Charbonnières les Bains,
134 France) and further sequenced on both strands with M13 universal primers (BigDye
135 Terminator v1.1 Sequencing Kit, Applied Biosystems) on an ABI PRISM 3130XL
136 genetic analyzer (Applied Biosystem, Courtaboeuf, France).

137 Sequences were compared with those deposited in the GenBank database
138 using NCBI's online BLAST2 program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

141 **RESULTS**

143 **Haemodialysis patients**

144 Among the 185 samples tested, 30.8% (n=57) were positive for PARV4 DNA
145 using the real-time approach described. Analysis of serological markers revealed that
146 PARV4 DNA was detectable in 27.3% (6/22) and 38.7% (29/75) of patients positive

for HCV and HBV, respectively. PARV4 DNA was further identified in 60% (3/5) of patients positive for HBsAg, but not in any of the samples positive for HIV (n=4). The titre of PARV4 DNA in the positive samples was low (<500 copies/ml of plasma).

No specific correlation was found between PARV4 viraemia and duration of dialysis or other available biological/clinical markers.

Lung transplant patients

PARV4 DNA was detected in 13.5% plasma samples (14/104), including one of the two patients with HCV positive pretransplant viraemia who received 24 weeks treatment with pegylated interferon alfa plus ribavirin, with a rapid virological response and a negative viraemia before transplantation. A low titre of PARV4 DNA (<500 copies/ml) was also noted. Patients with positive sera for PARV4 DNA were transplanted for cystic fibrosis (n= 8), emphysema (n=1), alpha 1 antitrypsin deficiency (n=1), pulmonary fibrosis (n= 3), sarcoidosis (n=1).

Dating of positive samples ranged from 1 (day of transplant procedure) to 1097 days after the allograft with a median of 206 days. No other serological marker was identified in the cohort studied. No difference in clinical outcome and survival was noted between recipients positive for PARV4 and those who were negative.

In order to analyse tentatively the time-course of infection in relation to patients positive for PARV4 DNA, additional serial plasma samples obtained at ± 6 months, with respect to the date of initial positive detection, were investigated when available. In each case (n=3, on average), attempts to detect PARV4 DNA in these samples remained unsuccessful.

Real-time PCR approach directed toward HBoV DNA gave no positive signal in any of the samples tested.

Genetic variability

Positive real-time PARV4 amplification products selected at random (n=15 and n=14 for haemodialysis patients and lung transplant recipients cohorts, respectively) were confirmed by analysis on agarose gel electrophoresis, whereas negative samples did not shown any amplification pattern. Amplification products confirmed their PARV4 origin following cloning and sequencing. Most of them exhibited 100% nucleotide identity with the PARV4 prototype isolate (GenBank accession no. AY622943) in this region, while three samples exhibited a point mutation (Fig. 1).

Absence of amplification was confirmed for each of the negative real-time HBoV amplification products analyzed.

DISCUSSION

The real distribution and implication of PARV4 in humans is still under investigation. Using the same molecular approach, the detection of PARV4 in about 20% of individuals without proven pathology like blood donors was reported recently, suggesting a non-negligible dispersion of the virus in the population [Touinssi et al., 2010]. This study demonstrates that about 31% of a cohort of patients in maintenance haemodialysis is infected with PARV4. Analysis of serological markers characterising the cohort tends to confirm that the prevalence of the virus would be higher in individuals showing, at least for HBV, hepatitis-related markers of infection.

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3 197 Such findings would be in accordance with the lower prevalence (13.5%) identified
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5 198 here in the cohort of lung transplanted patients, in which HCV, HBV and HIV markers
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8 199 were hardly absent due to the preliminary selection of patients undergoing lung
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10 200 allograft. However, the fact that this prevalence value was largely lower than that
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12 201 identified in the normal population was unexpected and possibly due to undiscovered
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15 202 aspects of PARV4 infection with respect to the immunosuppressive treatment of
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17 203 patients.

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19 204 HBoV DNA was not identified in the cohort of adult lung transplant recipients.
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22 205 This would confirm its preferential implication in respiratory diseases of young
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24 206 children, even if many aspects of its natural history remain to be clarified [Schildgen
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27 207 et al., 2008].

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29 208 A low titre of PARV4 viral particles (<500 copies/ml) was identified in all
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31 209 positive samples identified in both cohorts. This is an agreement with previous
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33 210 studies relating generally mild PARV4 levels in blood samples, although values
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35 211 reaching up to 10^6 copies/ml were reported as well [Fryer et al., 2006; Fryer et al.,
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37 212 2007; Biagini et al., 2008; Touinssi et al., 2010]. In addition, the presence of
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39 213 serological markers of HCV or HBV did not modify significantly the titre of viral
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41 214 particles of the positive samples in the study. It is conceivable, however, that
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43 215 fluctuations in viraemia would occur according to the immune status of the host, and
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45 216 that additional serial samples tested negative here in the case of lung transplant
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48 217 patients may represent periods of lowest replication of the virus, leading to
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50 218 undetectable levels in blood.

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52 219 It was also shown that point mutations may be identifiable in the short genomic
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55 220 zone dedicated to PARV4 detection. This is in agreement with a recent study
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58 221 demonstrating notable differences in the detection of the virus, even if two slightly
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different real-time approaches were used [Touinssi et al., 2010]. It is obvious that detection strategies correlate directly with the progressive estimation of the real genetic diversity of the virus. The fact that new, divergent, human and animal variants were identified recently highlights the need to optimize detection methods consistently.

This study demonstrates that haemodialysis and lung transplant patients could be infected by PARV4, extending the spectrum of viral infections identifiable in these cohorts [Alpers and Kowalewska, 2007; Fischer, 2008]. Many aspects characterizing PARV4 are still largely unknown: both natural history and implication in host health, along with its genetic diversity and real dispersion in human and animal species need to be address in a near future. Interestingly, some aspects of this virus would be close to those identified in another viral family identified thirteen years ago, *Anelloviridae* [Biagini, 2009], which biology remains largely misunderstood.

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LEGENDS

Fig. 1. Alignment of PARV4 partial sequences showing location of point mutations in the zone of real-time detection. HDx and LTx : sequences identified from haemodialysis and lung transplant patients, respectively (GenBank accession nos HM802325-27). Grey area : nucleotide sequence targeted by the real-time probe. Lowercase letters : 5'/3' ends of real-time primers.